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Complete Sequence and Characterization of the Channel Catfish Mitochondrial Genome

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In order to support analysis of channel catfish populations and genetic improvement programs, the channel catfish, *Ictalurus punctatus*, mitochondrial genome was completely sequenced and revealed gene structure and gene order common to vertebrates. Nucleotide sequence comparisons of cytochrome b (*Cytb*) and cytochrome c oxidase subunit 1 (*COI*) demonstrated genetic separation of the genera *Ictalurus*, *Pylodictis* and *Ameiurus* consistent with the taxonomic classification within Ictaluridae. The ictalurid *Cytb* nucleotide sequences were significantly different from a putative channel catfish *Cytb* sequence in GenBank. Genetic relationships based on mitochondrial DNA sequences indicated the value of channel catfish in genomic comparisons between teleosts. Pairwise alignment of DNA sequences revealed conservation of regulatory sequences in the D-loop region with other vertebrates. Analysis of D-loop sequences in commercial populations and a research strain revealed 28 polymorphic sites and 33 D-loop haplotypes. Sequence analysis revealed clustering of haplotypes within commercial farms and the USDA103 research line, but D-loop haplotypes were not sufficient to discriminate the USDA103 fish from commercial catfish.

Keywords: Catfish; Mitochondrial DNA; D-loop; Polymorphism

Database Accession No: NC_003489; AF484158-AF484165; AF482987

INTRODUCTION

The channel catfish, *Ictalurus punctatus*, is a freshwater species native to North America east of the Rocky mountains. Channel catfish serve as functional models in several areas of biological research because they adapt readily to culture and are large enough to provide sufficient amounts of tissue. Their immune system is one of the best characterized for any teleost (Miller *et al.*, 1998; Shen *et al.*, 2001). They are used in studies of sensory biology, environmental monitoring and toxicology (Caprio *et al.*, 1993; Martin and Black, 1998; Ploch *et al.*, 1998). Channel catfish have been distributed worldwide for sport fishing, but their foremost application is in aquaculture (Tucker, 2000).

Production of catfish for human consumption is currently the largest sector (47%) of commercial finfish production in North America (FAO, 2002), with 1200 commercial operations and 79,500 ha of production ponds in the United States. Production doubled from 1988 to 1998 and the U.S. catfish industry now processes 600 million pounds annually (USDA, 2002). The lack of sustained genetic selection in channel catfish has effectively maintained a high level of phenotypic variation in commercial and research populations (Bondari, 1984; Tave, 1986; Dunham and Smitherman, 1987; Wolters and Johnson, 1995) and genetic improvement programs leading to improved catfish lines are only beginning to be applied. However, catfish in ponds are difficult to observe and methods must be improved for the efficient identification of families and strains due to the similar physical characteristics of channel catfish.

Currently, catfish genetics researchers lack a comparative genetic linkage map that would increase the efficiency of broodstock selection. The current genetic linkage map consists mainly of

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non-conserved microsatellite markers (Waldbieser *et al.*, 2001). Current efforts to add conserved genes to the linkage map include sequences from cDNA libraries (Nonneman and Waldbieser, unpublished) and cloned gene sequences. However, more information is needed to determine the relationship of channel catfish with other teleosts in order to identify candidate organisms for comparative mapping.

The present research was designed to sequence and characterize the mitochondrial genome in support of catfish genetic improvement programs. Mitochondrial sequences were used in phylogenetic comparisons and to screen for mitochondrial sequence contamination in cDNA libraries. Variation in mitochondrial DNA sequences has been used to characterize fish populations (Ferris and Berg, 1987; Whitmore *et al.*, 1992; Cronin *et al.*, 1993; Cantatore *et al.*, 1994; Murdoch *et al.*, 1994), and the D-loop region contains the highest levels of DNA sequence variation (Ferris and Berg, 1987; Meyer, 1993). Therefore, we used D-loop sequence polymorphism in an initial effort to discriminate a recently introduced genetic line of catfish from fish currently in commercial production.

MATERIALS AND METHODS

DNA Isolation

Ictalurid blood samples were obtained from juvenile and adult channel catfish (*I. punctatus*), blue catfish (*Ictalurus furcatus*), yellow bullhead (*Ameiurus natalis*), white catfish (*Ameiurus catus*) and flathead catfish (*Pylodictis olivaris*) maintained at the Thad Cochran National Warmwater Research Center. Species identification was verified by taxonomic classification (Eddy, 1969). Genomic DNA was isolated from whole blood using DNAzol BD (Molecular Research Center, Inc., Cincinnati, OH). Whole fry, up to 7 days post-hatch, were obtained from six commercial operations in Mississippi and Arkansas and from the USDA Catfish Genetics Research Unit hatchery and stored at $-20°$ in 95% ethanol. Genomic DNA was prepared from rehydrated fry by alkaline/heat denaturation (Waldbieser and Bosworth, 1997).

Amplification and DNA Sequencing

Oligonucleotide primers used for PCR amplification of the channel catfish D-loop region were designed from the flanking threonine and phenylalanine tRNA genes of the common carp (*Cyprinus carpio*) mitochondrial genome (Chang *et al.*, 1994), and the 16S rRNA gene primers were designed from the flanking valine and leucine tRNA genes of this species. All primers (Table I) were synthesized by ResGen (Huntsville, AL). Mitochondrial DNA was amplified using ThermalAce Polymerase (Invitrogen Corporation, Carlsbad, CA) according to manufacturer's instructions. The D-loop and 16S rRNA products were amplified from a female channel catfish and cloned into pCR4Blunt-TOPO (Invitrogen). Plasmid clones were sequenced with ABI PRISM® BigDye™ Terminators chemistry on an ABI PRISM® 3700 DNA Analyzer (Applied Biosystems, Foster City, CA) in the USDA, ARS, Mid-South Area Genomics Laboratory. Subsequently, the complete mitochondrial genome was amplified in six overlapping fragments using oligonucleotide primers designed from sequences of the D-loop and 16S rRNA and partial sequences of cytochrome b (*Cytb*), cytochrome c oxidase subunit I (*COI*), cytochrome c oxidase subunit III (*COIII*) and NADH dehydrogenase subunit 5 (*ND5*) obtained from a channel catfish brain cDNA library (Nonneman and Waldbieser, unpublished). Fragments were amplified and cloned as above using primer sets MT1 + MT3, MT4 + MT6, MT5 + MT8, MT7 + MT10, MT9 + MT12 and MT2 + MT11. Cloned fragments were sequenced as above using the EZ::TN ⟨TET-1⟩ transposon (Epicentre Technologies, Madison, WI). Gaps in the mitochondrial genome contig were closed by amplification of fragments using MT4 + MT20, MT17 + MT18 and MT7 + MT27 and direct sequencing of PCR products (Smith *et al.*, 2001). The *COI* and *Cytb* genes of the other ictalurid species were PCR-amplified from genomic DNA using primers MT28 + MT29 and MT32 + MT33, respectively, and fragments were cloned into pCR4Blunt-TOPO and sequenced as above. Both strands were sequenced for all fragments. Plasmid subclones containing overlapping regions of the mitochondrial genome were stored as frozen bacterial stocks at $-80°$.

DNA Sequence Analysis

Sequence analysis and contig assembly were performed using SeqMerge (Accelrys, Madison, WI). Predicted tRNA sequences were identified using tRNAscan-SE (Lowe and Eddy, 1997), and RNA secondary structure was visualized using RNADraw version 1.1b2 (Matzura and Wennborg, 1996). Codon usage was determined using Codon-Frequency (Accelrys), and nucleotide frequency at each codon position was determined using MEGA version 2.1 (Kumar *et al.*, 2001).

Mitochondrial gene and genome sequences from non-ictalurid fish were obtained from GenBank (Table II). Conserved sequences in the D-Loop regions were identified by pairwise alignments using GAP (Accelrys) and manual adjustment to maximize sequence alignment. Genomic sequences encoding the 13 mitochondrial proteins were

TABLE I Primers used for PCR amplification and sequencing

Primer	Start position	Strand	Sequence	Gene/region
DF	16380	+	CATCGGTCTTGTAATCCG	$tRNA^{THR}$
DR	907	−	GCATCTTCAGTGCTATGC	$tRNA^{PHE}$
16F	1942	+	TACACCGAGAAGACATCC	$tRNA^{VAL}$
16R	3647	−	TTTACCATGCTCTGCCAC	$tRNA^{LEU}$
16a	2382	+	AATAGAAGTTCAGCCTCGTAC	16S rRNA
16b	2892	+	AGGAACTCGGCAAACATAAG	16S rRNA
16c	3264	+	GATACACCTCTAAGTCGCAG	16S rRNA
MT1	16398	+	CAGACTGAAGGTTCGACTCC	$tRNA^{THR}$
MT2	870	−	CTACAACAGCTATACAATATGATGTG	D-Loop
MT3	2084	−	CTTTGTGGTGCCCTTTCTGTC	16S rRNA
MT4	3458	+	AAGTCCTACGTGATCTGAGTTC	16S rRNA
MT5	6880	+	CCTATTTGTCTGAGCCGTCC	*COI*
MT6	7536	−	GTGTCGTGTATTGTGTATCCTG	*COI*
MT7	10113	+	AGGAGAACGCAAACAAGCAG	*COIII*
MT8	10273	−	GTTGAGCCGATGATGACGTG	*COIII*
MT9	13064	+	TGACCATTACTCACTGATTTTCACC	*ND5*
MT10	13270	−	TTCAACCGATTAGTAGGAAGG	*ND5*
MT11	15368	+	AGCTATGCACTACACCTCAGAC	*Cytb*
MT12	15452	−	CGTTAGCGTGGAGGTTGCGG	*Cytb*
MT17	2002	+	CCTAAATACCTAAAACAACATTACAAC	16S rRNA
MT18	4464	−	CATGTGGAAAGTGCATTGCTC	*ND1*
MT19	4380	+	CCGGAGGTCCTTTCGCAC	*ND1*
MT20	5644	−	AGCTCTCCTAGGGATAGTAG	*ND2*
MT25	10451	+	TTAAAGTTAGTACGAATGACTTCC	$tRNA^{GLY}$
MT26	11712	−	GTTTTGGTGTAGGAGGAGCAG	*ND4*
MT27	12083	−	TGTGGCTGACGGATGAGTAG	*ND4*
MT28	15164	+	TCTAACCAGGACTAATGACTTGAA	$tRNA^{GLU}$
MT29	16409	−	ACCTTCAGTCTCCGGATTAC	$tRNA^{THR}$
MT30	15732	+	GCAACACTAACCCGATTCTTC	*Cytb*
MT31	15842	−	GATTGGGTTGTTAGAGCCTG	*Cytb*
MT32	6281	+	AAACCTTTGTTCATGGAGCTAC	$tRNA^{TYR}$
MT33	7943	−	AGTGGTTATGTAACTGGCTTGA	$tRNA^{SER}$
MT34	7047	+	TTGGGCACCCAGAAGTATAC	*COI*
MT35	7158	−	CAGACTATTCCCATATAGCCG	*COI*

TABLE II Mitochondrial gene and genome accessions of species used in present research

Superorder/Order	Family	Scientific name	Common name	GenBank Accession
Elopomorpha				
Anguilliformes	Anguillidae	*Anguilla japonica*	Japanese eel	NC_002707
	Congridae	*Conger myriaster*	Conger eel	NC_002761
Ostariophysi				
Siluriformes	Ictaluridae	*Ictalurus punctatus*	Channel catfish	NC_003489*
		Ictalurus furcatus	Blue catfish	AF484165*, AF484159*
		Pylodictis olivaris	Flathead catfish	AF484160*, AF484161*
		Ameiurus catus	White catfish	AF484162*, AF484163*
		Ameiurus natalis	Yellow bullhead	AF484164*, AF484158*
	Cranoglanidae	*Cranoglanis bouderius*	Helmet catfish	AF475155
	Bagridae	*Hemibagrus guttatus*	Lang	AF475154
	Clariidae	*Clarias fuscus*	Whitespotted clarias	AF475157
		Clarias gariepinus	North African catfish	AF475153
Cypriniformes	Cyprinidae	*Cyprinus carpio*	Common carp	NC_001606
		Carassius auratus	Goldfish	NC_002079
		Danio rerio	Zebrafish	NC_002333
Protacanthopterygii				
Salmoniformes	Salmonidae	*Salmo salar*	Atlantic salmon	NC_001960
		Oncorhynchus mykiss	Rainbow trout	NC_001717
Acanthopterygii				
Tetraodontiformes	Tetraodontidae	*Takifugu rubripes*	Pufferfish	NC_004299

*Sequence obtained in present research.

concatenated and aligned using PILEUP, and pairwise genetic distances were determined using the Kimura 2-parameter model with 1000 bootstrap iterations (Kumar *et al.*, 2001).

Phylogenetic relationships were determined by maximum parsimony (MP) and maximum likelihood (ML) analyses on Siluriform *Cytb* and *COI* gene sequences, using Cyprinids as an outgroup. Maximum parsimony analyses were performed using a heuristic search with 10 random sequence additions and included all positions. A total of 1000 bootstraps were used to generate the consensus tree (Kumar *et al.*, 2001). For the maximum likelihood analysis, trees were constructed with fastDNAml version 1.2 (Felsenstein, 1981; Olsen *et al.*, 1994) using global rearrangement. Maximum likelihood trees calculated in fastDNAml were generated using the program TreeView version 1.6.6 (Page, 1996).

cDNA Library Screening

cDNA sequences were obtained by 5′ end sequencing of cDNA libraries derived from channel catfish brain and cultured immunocytes (unpublished data). Base calling was performed using Phred v0.000925.c (Ewing *et al.*, 1998; Ewing and Green, 1998). Sequences were processed with Lucy (Chou and Holmes, 2001) to remove vector sequence and delete clones with less than 100 Phred20 bases. These sequences were compared with the complete mitochondrial genome using BlastN (Altschul *et al.*, 1990).

Mitochondrial D-loop Polymorphism

The D-loop region was amplified from full-sib offspring of a channel catfish X blue catfish mating and a reciprocal mating using primers DF and DR (Table I). After thermal cycling, 1 unit *Mbo*1 (New England Biolabs, Beverly, MA) was added directly to the reaction mix, products were digested 1 h at 37°, separated on a 2.5% agarose gel, and visualized with ethidium bromide. Sequence polymorphism in the D-loop region was determined by amplification of fry DNA with primers MT1 + MT2 and direct sequencing of the amplification product with these primers (Smith *et al.*, 2001). Fry were obtained from 67 full-sib families of the USDA103 strain and from random, mixed spawns collected from six commercial farms. D-loop sequence polymorphism was verified by sequence alignment and chromatogram analysis with SeqMerge. Prepared samples from farms were archived at −20° and non-processed fry were stored in ethanol at −20°. Haplotype diversity and analysis of molecular variance were calculated using Arlequin version 2.0 (Schneider *et al.*, 2000).

RESULTS

We determined the complete sequence of the channel catfish mitochondrial genome by transposon-mediated DNA sequencing of cloned PCR fragments, direct sequencing of PCR fragments from selected regions and assembly of sequence data into a single contig of 16,497 bp. Sequence analysis revealed genes encoding 13 proteins, 2 ribosomal RNAs and 22 transfer RNAs (Table III) with gene order and strand orientation common to vertebrates (Boore, 1999). The largest intergenic regions were 30 bp between $tRNA^{ASN}$ and $tRNA^{CYS}$ and 15 bp between the $tRNA^{ASP}$ and the start of *COII*. The former intergenic region contained sequence with the potential to form a stable stem-loop structure and was a common location for the vertebrate origin of light strand replication (Shadel and Clayton, 1997). Protein-encoding sequences overlapped by 10 bp at the end of *ATP8* and beginning of *ATP6*, 7 bp at the end of *ND4L* and beginning of *ND4* and 4 bp at the end of both *ND5* and *ND6*. Sequence information from clones in a channel catfish brain cDNA library verified polyadenylation of the 3′ end of the *COIII* and *Cytb* transcripts to produce a stop codon (TAA). The *COII* and *ND3* reading frames ended with a T in the 1st position of a putative stop codon and polyadenylation to form a TAA stop codon was inferred from comparison with the other species in this study.

Nucleotide composition and codon frequencies were calculated from the 13 gene contig. A Chi-square goodness-of-fit test on nucleotide composition revealed no bias ($P < 0.05$) at the 1st codon position, bias against *A* and *G* and toward T at the 2nd position, and bias toward *A* and *C* and strongly against *G* in 3rd position (Table IV). Except for methionine, codons for amino acids with a corresponding tRNA in the mitochondrial genome always ended in *A* or *C* (Table V). The bias at the 3rd codon position was evident in all amino acids except isoleucine ($T > C$), valine ($A > T > C$) and phenylalanine ($T = C$). Two stop codons in the vertebrate mitochondrial genome, AGA and AGG, were not used in any catfish mitochondrial genes.

Pairwise alignment of D-loop sequences identified several regions of conserved sequence between selected teleosts (Table II), although the repetitive nature of the conger eel D-loop region led to no useful consensus alignments. Catfish D-loop sequence between bases 630 and 808 was similar to conserved sequence blocks CSB1, CSB2 and CSB3 (Table VI) associated with heavy strand replication in vertebrate mitochondrial genomes (Walberg and Clayton, 1981; Sbisà *et al.*, 1997). Sequence similarity between teleosts was high at CSB2 and CSB3, but low in the region similar to the consensus mammalian CSB1 (Sbisà *et al.*, 1997). Teleost CSB1's were more

TABLE III Catfish mitochondrial genes

Gene	Abbrevation	Start	End	Codon recognized	Amino acids
Displacement loop (control region)	D-loop	1	885		
$tRNA^{PHE}$	F	886	955	TTC	
12S ribosomal RNA	12S	956	1908		
$tRNA^{VAL}$	V	1909	1980	GTA	
16S ribosomal RNA	16S	1981	3640		
$tRNA^{LEU}$	L	3641	3715	TTA	
NADH dehydrogenase subunit 1	*ND1*	3716	4690		324
$tRNA^{ILE}$	I	4692	4763	ATC	
$tRNA^{GLN}$	Q	4833	4763	CAA	
$tRNA^{MET}$	M	4833	4902	ATG	
NADH dehydrogenase subunit 2	*ND2*	4903	5949		348
$tRNA^{TRP}$	W	5948	6018	TGA	
$tRNA^{ALA}$	A	6089	6021	GCA	
$tRNA^{ASN}$	N	6163	6091	AAC	
$tRNA^{CYS}$	C	6261	6195	TGC	
$tRNA^{TYR}$	Y	6333	6264	TAC	
Cytochrome c oxidase subunit 1	*COI*	6335	7885		516
$tRNA^{SER}$	S	7956	7886	TCA	
$tRNA^{ASP}$	D	7961	8033	GAC	
Cytochrome c oxidase subunit 2	*COII*	8048	8738		230*
$tRNA^{LYS}$	K	8739	8812	AAA	
ATP synthase F0 subunit 8	*ATP8*	8814	8981		55
ATP synthase F0 subunit 6	*ATP6*	8972	9655		227
Cytochrome c oxidase subunit 3	*COIII*	9655	10438		261*
$tRNA^{GLY}$	G	10439	10510	GGA	
NADH dehydrogenase subunit 3	*ND3*	10511	10861		116
$tRNA^{ARG}$	R	10860	10929	CGA	
NADH dehydrogenase subunit 4L	*ND4L*	10930	11226		98
NADH dehydrogenase subunit 4	*ND4*	11220	12600		460*
$tRNA^{HIS}$	H	12601	12670	CAC	
$tRNA^{SER}$	S	12673	12736	AGC	
$tRNA^{LEU}$	L	12812	12740	CTA	
NADH dehydrogenase subunit 5	*ND5*	12813	14636		607
NADH dehydrogenase subunit 6	*ND6*	15148	14633		171
$tRNA^{GLU}$	E	15217	15149	GAA	
Cytochrome B	*Cytb*	15219	16356		379*
$tRNA^{THR}$	T	16357	16428	ACA	
$tRNA^{PRO}$	P	16497	16427	CCA	

*TAA stop codon is completed by the addition of 3′ A residues to mRNA.

similar within taxonomic families than between, and the catfish and cyprinid sequences were more similar to the consensus mammalian CSB1 than were the salmonid CSB1 regions. Among teleosts, there was more similarity in the region (bases 651–691) adjacent to CSB1 than within the consensus CSB1. Sequence from bases 322 to 555 of the catfish genome was similar to the mammalian D-loop central domain (Sbisà *et al.*, 1997), and several regions in this domain were conserved among the teleosts (Table VII). The D-loop sequence from bases 45 to 92 contained a potential stem-loop structure containing two TACAT motifs common to mammalian sequences associated with the arrest of heavy strand synthesis (Doda *et al.*, 1981; Dunon-Bluteau and Brun, 1987; Sbisà *et al.*, 1997).

TABLE IV Nucleotide frequencies (percent) by codon position over all mitochondrial protein-encoding genes

	A	C	G	T
1st position	26.5	26.1	25.8	21.6
2nd position	18.6	27.1	13.5	40.8
3rd position	36.0	33.8	8.5	21.7
Total	27.1	29.0	15.9	28.0

Alignment and comparison of all 13 protein-encoding genes showed that pairwise genetic distances (Table VIII) between catfish and common carp, goldfish, Atlantic salmon and rainbow trout were similar (average = 0.23). Channel catfish were more similar to zebrafish (0.27) than to pufferfish (0.35). Phylogenetic analyses of the siluriform *Cytb* sequences using maximum parsimony generated the expected topology based on established taxonomic relationships (Table II). Maximum likelihood analysis resulted in a log likelihood value of 7289.99, with nucleotide frequencies of $A = 0.2896$, $C = 0.2897$, $G = 0.1368$ and $T = 0.2839$. The overall topology of the optimal tree produced under this model (Fig. 1) was consistent with those produced by the maximum parsimony analyses. All node assignments were significant ($P < 0.01$). There was strong support for the branching of Siluriformes from Cypriniformes, and a separation of the families Ictaluridae, Cranoglanidae, Bagridae and Clariidae within Siluriformes. Genera within Ictaluridae were

TABLE V Codon usage in channel catfish mitochondrial protein-encoding genes

Amino acid	Codon*	Number	Frequency per 1000	Codon usage (%)
Lys	AAA*	67.00	17.61	84
	AAG	13.00	3.42	16
Asn	AAC*	73.00	19.19	61
	AAU	47.00	12.35	39
Thr	ACA*	110.00	28.91	37
	ACG	9.00	2.37	3
	ACC	123.00	32.33	42
	ACU	52.00	13.67	18
Stop	AGA	0.00	0.00	0
	AGG	0.00	0.00	0
Ser	AGC*	37.00	9.72	16
	AGU	9.00	2.37	4
Met	AUA	113.00	29.70	65
	AUG*	59.00	15.51	35
Ile	AUC*	118.00	31.01	40
	AUU	179.00	47.04	60
Gln	CAA*	93.00	24.44	93
	CAG	7.00	1.84	7
His	CAC*	78.00	20.50	71
	CAU	31.00	8.15	29
Pro	CCA*	68.00	17.87	33
	CCG	8.00	2.10	4
	CCC	99.00	26.02	47
	CCU	34.00	8.94	16
Arg	CGA*	43.00	11.30	58
	CGG	6.00	1.58	8
	CGC	19.00	4.99	26
	CGU	6.00	1.58	8
Leu	CUA*	254.00	66.75	40
	CUG	51.00	13.40	8
	CUC	103.00	27.07	16
	CUU	92.00	24.18	14
Glu	GAA*	81.00	21.29	85
	GAG	15.00	3.94	15
Asp	GAC*	49.00	12.88	63
	GAU	29.00	7.62	37
Ala	GCA*	99.00	26.02	29
	GCG	15.00	3.94	4
	GCC	174.00	45.73	51
	GCU	54.00	14.19	16
Gly	GGA*	83.00	21.81	34
	GGG	49.00	12.88	20
	GGC	86.00	22.60	35
	GGU	26.00	6.83	11
Val	GUA*	85.00	22.34	38
	GUG	30.00	7.88	14
	GUC	46.00	12.09	21
	GUU	61.00	16.03	27
Stop	UAA	10.00	2.63	77
	UAG	3.00	0.79	23
Tyr	UAC*	64.00	16.82	56
	UAU	49.00	12.88	44
Ser	UCA*	61.00	16.03	26
	UCG	7.00	1.84	3
	UCC	81.00	21.29	35
	UCU	38.00	9.99	16
Trp	UGA*	92.00	24.18	75
	UGG	30.00	7.88	25
Cys	UGC*	17.00	4.47	63
	UGU	10.00	2.63	37
Leu	UUA*	112.00	29.43	18
	UUG	22.00	5.78	4
Phe	UUC*	118.00	31.01	52
	UUU	108.00	28.38	48

* tRNA with anticodon present in mitochondrial genome.

TABLE VI Alignment of conserved sequence blocks (CSB) in the mitochondrial D-loop region*

	630 **CSB1** 650
Catfish	AAAAATGTAATGCTTT-AATGA
Carp	T.T.TAT....TA.CG-T.A..
Goldfish	T.T..ATG...TA.CG-T.A..
Zebrafish	T.T.TAT....TA.C.-..A..
Salmon	-CCC.....TCATGGT-G-GA.
Trout	.CCC.....TCATGGT-G.AAT
Pufferfish	GGT.....T.ATTAA.G...T.
Japanese eel	T...G.TG....T.AG-..A..

	651 691
Catfish	CATACTTG-AAATAA-TTACATAACTCTC-TCTCAAGTACATAA
Carp	....A.-T-T..G..-.....-..T.TA.....G.....
Goldfish	....A.-T-T..G..-.....-..TCTAA.....G.....
Zebrafish	....A.AT-T..G.C-.C..-.....T.TAAT.....G.....
Salmon	G...T.CTAT..AG.A.C.....CTTGGA-TA.....G.....
Trout	G...T.CTAT..AG.A.C.....CTTGGA-TA.....G.....
Pufferfish	AGACA..ATTT.AG.A.....TA..--GA.A.....G.....
Japanese eel	...TAAAT-.....-CC....T.AAATA-.A.....G.....

	740 **CSB2** 763
Catfish	TGCGCGGTAAACCCCCC-TACCCCC
Carp	......AC.....T.....
Goldfish	......AC.....-.....
Zebrafish	......AC.....T-.....
Salmon	.....C.....-.....
Trout	.....-.....
Pufferfish	ATTAAC...T.....C.....
Japanese eel	.T--..TC.....-.....

	786 **CSB3** 808
Catfish	TCC-TGTTAAACCCCTAAACCA-GG
Carp	...T...C.....G.....A..
Goldfish	...T...C.....G.....A..
Zebrafish	...T...C.....A..
Salmon	...-...C.....-..
Trout	...-.....-..
Pufferfish	.AA-GT..G....AAGCTCT.C-AC
Japanese eel	...-...C.....-..

*Numbers represent position in channel catfish mitochondrial genome. Dots represent nucleotide identical to catfish sequence. Dashes represent gaps.

separated according to taxonomic classification, and this was supported by analyses using *COI* data (not shown). The *Cytb* sequence from a putative channel catfish propagated in Japan (Matsuo *et al.*, 2001, GenBank ID: AB045119) was included in the analyses. The pairwise genetic distance between the Japanese *Cytb* sequence and the known ictalurid sequences ranged from 0.083 to 0.094 (±0.012), while its distance from the common carp and goldfish was only 0.015 to 0.03 (±0.006). Both the maximum likelihood and maximum parsimony analyses grouped this putative catfish sequence with the cyprinids.

Sequences obtained from 8061 clones from brain and cultured immunocyte cDNA libraries were screened for mitochondrial transcript contamination using BlastN against the entire mitochondrial genome. Three to six percent of the clones in each library contained mitochondrial transcripts (289 total). The most abundant transcript in each library was *COI* (37–60%), but transcripts were detected

TABLE VII Alignment of conserved sequences in the central domain of the mitochondrial D-loop region*

	312 328	355 371
Catfish	AGTAAGAGACCACCATC	TTGAACGA-TCAGGGACA
Salmon	.....AC.G....A.	....TG.--.....
Trout	.....AC.G....A.	....TG.--.....G..
Carp	G.....A.	A...TA..A...A.....
Goldfish	.....A.....A.	A...TG..A...A.....
Zebrafish	...G.....A-	A...TA..A.....
Pufferfish	A.....AC.G.....	.....G.--.G.....
Japanese eel	.....A.....A.	A...TAGG-.....

	395 428
Catfish	TGCACTATTACTGGCATCTGGTTCCT-ATTTCAGG
Salmon	..A.....C.....T.....ATA.CA...
Trout	..A.T....C.....T.....A.G.CA...
Carp	..A.....C.....
Goldfish	..A.....-.....
Zebrafish	..A.....C....T.....-.AA..C...C.
Pufferfish	..A.....C.....T.....-.C.....
Japanese eel	..A.....T.....-.....

	502 538
Catfish	ACCCCCCAAGCCAAGGCGTTCTTTTAT-AGGCATGGGGT
Salmon	....A.....G-.....C....-.T....A....
Trout	....A.....G-.....C....-.T....A....
Carp	.....T...G..-.A.....-.T....A....
Goldfish	.....A..T...G..-.A.....-.T....AAC..
Zebrafish	..T.....T...G..-.....G....-.TA..A.....
Pufferfish	.AT...AC.TG.CG.....C.CCAC...GG.CA...
Japanese eel	....A.....GG.-.A..AAC...A-.....TT...

*Numbers represent position in channel catfish mitochondrial genome. Dots represent nucleotide identical to catfish sequence. Dashes represent gaps.

from every protein-encoding gene (Table III) as well as from the 12S rRNA and 16S rRNA genes.

The D-loop region was used to identify DNA sequence polymorphism in channel catfish populations. The channel catfish D-loop region contained two *MboI* restriction sites at bases 361 and 722. However, a nucleotide substitution in the blue catfish D-loop region at base 722 abolished one *MboI* site. Therefore, amplification of template DNA with primers DF and DR (Table I), and digestion with *MboI*, produced D-loop restriction fragments of 479, 361 and 185 bp from channel catfish and 546 and 479 bp from blue catfish (Fig. 2). Twenty full-sib offspring each from channel x blue hybrid matings

TABLE VIII Pairwise genetic distances calculated from nucleotide sequences of all mitochondrial protein-encoding genes (Kimura 2-parameter model on first 2 codon positions using bootstrap analysis with 1000 iterations). Average standard error = 0.0065

	Catfish	Carp	Goldfish	Zebrafish	Salmon	Trout	Fugu	Japan Eel
Common carp	0.231							
Goldfish	0.231	0.106						
Zebrafish	0.270	0.218	0.212					
Atlantic salmon	0.231	0.220	0.219	0.275				
Rainbow trout	0.235	0.223	0.222	0.285	0.100			
Pufferfish	0.349	0.323	0.328	0.368	0.318	0.325		
Japanese eel	0.240	0.222	0.224	0.262	0.220	0.232	0.340	
Conger eel	0.278	0.262	0.259	0.286	0.263	0.272	0.368	0.211

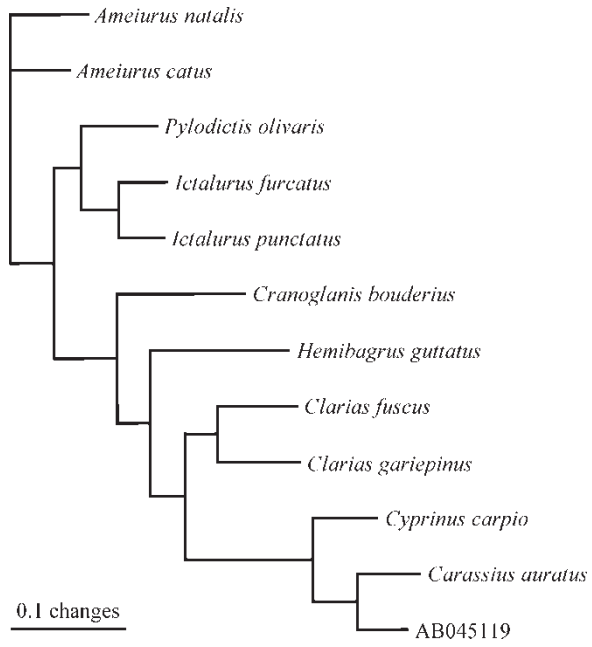

FIGURE 1 Maximum likelihood tree for nucleotide sequence comparisons of available cytochrome b sequences from the order Siluriformes. *Cyprinus carpio* and *Carassius auratus*, from the order Cypriniformes, are included as outgroups. GenBank accession AB045119 is putative channel catfish from Lake Kasumigaura, Japan.

were analyzed, and only maternal inheritance of the D-loop region was detected.

Analysis of 841 bp of the D-loop sequence revealed high levels of diversity among these catfish populations. The catfish populations in this research displayed 28 polymorphic sites (Table IX); 5 were insertion/deletions (17.8%), 18 were transitions (64.3%) and 5 were transversions (17.8%) to produce a total of 33 haplotypes. Seventy-two catfish from six commercial farms contained 30 of the haplotypes, and 3 haplotypes (2,3 and 4) were unique to the USDA103 families (Fig. 3). Farm haplotypes 5, 16, 20 and 22 were found in 47% of the commercial fish, but the remaining haplotypes were represented by only 1 or 2 fish within a farm. Only seven haplotypes (5, 7, 16, 20, 22, 25 and 26) were shared between commercial catfish farms, usually between only two farms. Only haplotype 5 was found in more than two farms, and it was also present in one USDA103 family. In the USDA103 strain, 64 of 67 families displayed only haplotypes 1 and 2. Of the remaining three USDA103 families, two displayed unique haplotypes (2 and 3) that were not shared with commercial fish and one family displayed haplotype 5.

TABLE IX Mitochondrial D-loop haplotypes in channel catfish populations

	Position (bp)																											
	3	55	61	69	92	96	97	98	99	104	108	114	115	138	160	167	279	298	305	311	330	332	381	484	605	656	684	730
Hap[a]	T	G	I/D	C	A	T	A	T	T	C	I/D	I/D	T	C	T	T	T	T	T	C	T	G	A	A	C	T	I/D	C
1	·	·	–	·	·	·	·	·	·	·	–	–	·	T	·	·	·	·	·	·	·	·	·	·	·	·	–	·
2	·	·	–	·	·	·	·	·	·	·	–	–	·	T	C	·	·	·	·	·	C	A	·	·	·	·	–	·
3	·	·	–	·	·	·	·	·	·	·	–	–	·	T	C	·	·	·	·	·	·	·	·	·	·	·	–	·
4	·	·	-	·	·	C	T	C	A	·	-	-	·	T	·	·	·	·	·	·	·	·	·	·	·	·	-	·
5	·	·	-	·	·	·	·	·	·	·	-	-	·	·	·	·	·	·	·	·	·	·	·	·	·	·	-	·
6	·	·	-	·	·	·	·	·	·	·	-	-	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·
7	·	·	C	·	·	·	·	·	·	·	–	–	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·
8	·	·	C	·	·	·	·	·	·	·	-	-	·	·		·	·		·	·	·	·	·	·	·	·	-	·
9	·	·	C	·	·	·	·	·	·	·	-	-	·	T	·	·	·	·	·	·	·	·	·	·	·	·	-	T
10	·	·	C	·	·	·	·	·	·	·	-	C	I/D	T	·	·	·		·	·	C	·	G	G	T	C	-	·
11	·	·	C	·	·	·	·	·	·	·	-	C	I/D	T	C	·	·	C	·	·	C	·	G	G	T	·	-	·
12	·	·	C	·	·	·	·	·	·	·	-	C	I/D	T	C	·	·	C	·	·	C	·	G	G	T	·	A	·
13	·	·	C	·	·	·	·	·	·	·	G	C	I/D	T	C	·	·	C	·	·	C	·	G	G	T	·	-	·
14	·	C	-	G	·	·	·	·	·	·	G	-	·	·	·	·	·	·	·	·	·	·	·	·	·	·	-	·
15	·	·	-	·	T	·	·	·	·	·	-	-	·	T	·	·	·	·	·	·	·	·	·	·	·	·	-	T
16	C	·	-	·	·	·	·	·	·	·	-	C	I/D	T	·	·	·	·	·	·	C	·	G	G	T	·	-	·
17	·	·	–	·	·	·	·	·	·	·	–	C	I/D	·	·	·	·	·	·	·	·	·	·	G	T	·	A	·
18	·	·	-	·	·	·	·	·	·	·	-	C	I/D	·	·	·	·	·	·	·	C	·	G	G	T	·	-	·
19	·	·	-	·	·	·	·	·	·	·	-	C	I/D	T	·	·	·	·	·	·	C	·	·	G	T	·	A	·
20	C	·	-	·	·	·	·	·	·	·	-	C	I/D	T	C	·	·	C	·	·	C	·	G	G	T	·	-	·
21	C	·	-	·	·	·	·	·	·	·	-	-	·	T	·	C	·	·	·	·	·	·	G	·	T	C	-	·
22	·	·	-	·	·	·	·	·	·	·	-	-	·	T	·	·	·	·	·	·	·	·	·	·	·	·	-	T
23	·	·	-	·	·	·	·	·	·	T	-	-	·	T		·	·	·	·	·	·	·	·	·	·	·	-	T
24	·	·	–	·	·	·	·	·	·	·	–	–	·	T	·	·	C	·	C	T	·	·	·	·	·	·	–	T
25	·	·	–	·	·	·	·	·	·	·	–	–	·	T	·	·	·	·	·	·	·	·	·	·	T	C	–	·
26	·	·	–	·	·	·	·	·	·	·	–	–	·	T	C	·	·	·	·	·	C	·	·	·	·	·	–	·
27	C	·	-	·	·	·	·	·	·	·	-	C	·	T	C	·	·	C	·	·	C	·	·	G	T	·	-	·
28	C	·	-	·	·	·	·	·	·	·	-	C	·	T	·	·	·	·	·	·	C	·	·	·	T	·	-	·
29	·	·	-	·	·	·	·	·	·	·	-	C	·	T	C	·	·	·	·	·	·	·	·	·	·	·	-	·
30	·	·	-	·	·	·	·	·	·	·	-	C	·	T	C	·	·	·	·	·	C	·	·	G	T	·	-	·
31	·	·	–	·	·	·	·	·	·	·	–	C	·	T	·	·	·	·	·	·	·	·	·	·	·	·	–	·
32	·	·	-	·	·	·	·	·	·	·	-	C	·	T	·	·	·	·	·	·	C	·	·	G	·	·	-	·
33	·	·	–	·	·	·	·	·	·	·	–	–	·	T	·	·	·	·	·	·	·	·	·	·	T	·	–	·

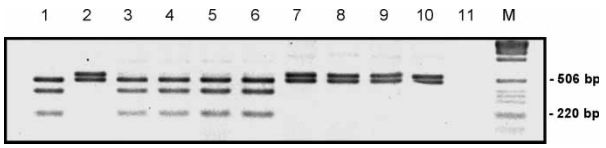

FIGURE 2 Inverse image of ethidium-stained agarose gel containing PCR-amplified D-loop fragment digested with *MboI* from channel catfish (lane 1), blue catfish (lane 2), four full-sib offspring from a channel female x blue male hybrid cross (lanes 3–6) and four full-sib offspring from a blue female x channel male hybrid cross (lanes 7–10). Lane 11 is a negative control PCR reaction with no genomic template and the marker lane (M) is 1 kb ladder.

Statistical analyses of haplotype diversity demonstrated marked differences between the USDA103 and farm populations. The USDA103 population displayed lower gene and nucleotide diversity than the farm populations (Table X). Calculated fixation indices (Table XI) demonstrated the farms, taken as a whole, were not different from the USDA103 population, but there were significant differences among farms (F_{SC}) and among all populations in the study (F_{ST}). The significant diversity was also reflected in the lack of distinct structure in the haplotype network (Fig. 3).

DISCUSSION

The channel catfish mitochondrial genome structure was typical of most vertebrates, with identical gene order, little extraneous DNA sequence, and transcript polyadenylation to form stop codons in four genes. The catfish bias toward A and C at the 3rd codon position was evident in all codons except for isoleucine and phenylalanine. Codons for which the anticodon was present in the mitochondrial tRNA gene were generally preferred, although this trend was more evident in codons with twofold degeneracy. Sequence similarities in the D-loop region with other vertebrates implied similar control of mitochondrial replication and transcription.

Channel catfish are grouped with cyprinids in the superorder Ostariophysii (Moyle and Cech, 1988; Miya and Nishida, 2000; Miya *et al.*, 2001; Elmerot *et al.*, 2002), which is more primitive than Protacanthopterygii (includes salmonids) and Acanthopterygii (includes pufferfish). However, mitochondrial pairwise genetic distances did not reflect these groupings because the channel catfish was more similar to Atlantic salmon and rainbow trout than to zebrafish. The significant differences in pairwise genetic distances between the ictalurid *Cytb* sequences and GenBank sequence AB045119 implied this was not from a channel catfish as had been reported (Matsuo *et al.*, 2001). The grouping of Genbank accession AB045119 with the cyprinids suggested a clerical error, perhaps during storage or submission, rather than a misclassification based on fish morphology.

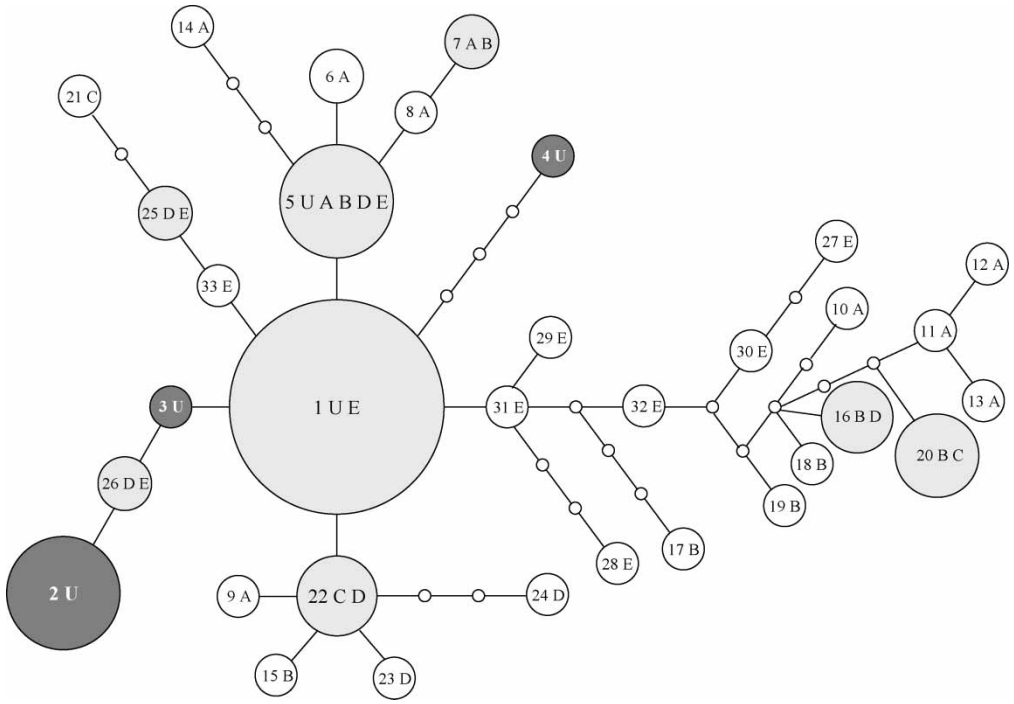

FIGURE 3 Network of channel catfish D-loop haplotypes from USDA103 and farm populations. Geneological relationships among haplotypes were estimated by the parsimony method described by Templeton *et al.* (1992). Numbers correspond to haplotype and letters correspond to population (U—USDA103, A to E—farm populations). Each line represents one mutation between haplotypes. White circles represent haplotype found in only one farm, light gray circles represent haplotypes shared between populations, dark gray circles represent haplotypes found only in the USDA103 population. Unlabeled white circles represent missing haplotypes.

TABLE X Gene and nucleotide diversity of D-loop haploptypes for USDA103 and farm populations of channel catfish. Variances are shown to the right of the measure of diversity

Population	Gene diversity	Variance	Nucleotide diversity	Variance
All samples	0.8053	0.0298	0.0032	0.0019
USDA103	0.4355	0.0580	0.0015	0.0011
Farm A	0.9697	0.0443	0.0052	0.0031
Farm B	0.8846	0.0699	0.0040	0.0025
Farm C	0.6889	0.1038	0.0058	0.0035
Farm D	0.7273	0.1444	0.0040	0.0025
Farm E	0.8182	0.0740	0.0023	0.0015

Comparison of genomic data with the complete nuclear genomes of the model species zebrafish and pufferfish will be useful in genetic analyses of aquacultured species. Researchers interested in catfish, carps and salmonids may find the zebrafish more useful than the pufferfish due to greater genetic similarity. The genetic distance measurements suggest zebrafish and pufferfish may not be representative of more primitive teleosts, so addition of genomic information from species such as channel catfish would add another dimension to comparative analyses of vertebrate genomes. Such molecular genetic analyses would be supported by a growing EST database (TIGR, 2003) and two bacterial artificial chromosome libraries (CHORI, 2003; Quiniou *et al.*, 2003) for channel catfish.

Verification of maternal inheritance from PCR-amplified DNA, and lack of detectable paternal contribution, provided the basis for screening catfish populations for mitochondrial haplotypes. Commercial fry were collected from hatching troughs that contained fry from multiple spawns. These haplotypes could reflect a limited ancestral pool of female broodfish on each farm and/or over-representation of certain spawns at the time of collection. Therefore, haplotype-based identification might be used to determine broodfish or families with greater reproductive success. Haplotype screening could also be used in the study of natural catfish populations and management of introduced catfish populations. There were significant differences in haplotype frequency between farms, but there was no clear haplotype structure within or between farms probably due to artificial selection and separation of populations for commercial production. The most prevalent USDA103 haplotype was observed in 41% of the samples of Farm E, and one USDA103 family had a haplotype shared with four farms. While channel catfish are native to the Mississippi River drainage system, the USDA103 strain was developed from catfish collected from the Colorado River drainage system (Wolters *et al.*, 2000) and most likely stocked there from a state or federal hatchery. Thus genetic differences observed between the USDA103 and commercial catfish populations probably reflected selective breeding more than geographical separation.

The complete mitochondrial genome sequence proved useful in confirming taxonomic relationships within Siluriformes, estimating genetic distances between channel catfish and other cultured teleosts and efficient removal of all mitochondrial contaminant clones in cDNA sequence databases. The current research identified a wealth of haplotype diversity in channel catfish populations that can be useful for analysis of cultured and natural populations. Haplotype analysis based on D-loop polymorphism was not useful in discriminating a newly introduced genetic line from existing commercial fish. Future selection of female broodstock to limit mitochondrial haplotypes would assist DNA fingerprinting, certification and management of genetically improved populations released to commercial producers. Sequence polymorphism in other genes, such as 16S rRNA, may be necessary to enhance the utility of mitochondrial haplotype screening in cultured catfish populations. The data from the current research support ongoing research in comparative genetic mapping and selective breeding of channel catfish for commercial production.

TABLE XI Summary of analysis of molecular variance (AMOVA) in D-loop haplotypes for USDA103 and farm populations of channel catfish. Levels of significance are based on 1023 permutations

Level of variation	df	Variance	%	Fixation index*	*P*
Among USDA103 and farms	1	0.0687	15.22	$F_{CT} = 0.15216$	NS
Among populations	4	0.0702	15.54	$F_{SC} = 0.18330$	<0.001
Within populations	129	0.3127	69.24	$F_{ST} = 0.27635$	<0.001

*F_{CT}, correlation among random haplotypes within each group (USDA103 or farms) relative to the correlation of random pairs drawn from the whole samples; F_{SC} correlation among random haplotypes within populations relative to the correlation of random pairs drawn from the group (USDA103 or farms); F_{ST} correlation among random haplotypes within populations relative to random pairs drawn from the whole sample.

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